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TITLE: Sildenafil and Phosphodiesterase-5 Inhibitors to Reduce Cardiotoxicity and Enhance the Response of Breast Tumors to Doxorubicin

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14. ABSTRACT In our studies of the interaction between sildenafil and adriamycin in breast tumor cells and cardiomyocytes, we have made the following observations. In breast tumor cells: 1. Sildenafil fails to protect various breast tumor cell lines against the toxicity of adriamycin. 2. Sildenafil moderately enhances the response to adriamycin in breast tumor cells. 3. Sildenafil does not alter the extent of DNA damage induced by adriamycin in breast tumor cells. 4. Adriamycin promotes autophagy in breast tumor cells. 5. Sildenafil appears to increase sensitivity to irradiation in breast tumor cells. In cardiomyocytes: 1. Questions are raised relating to the role of reactive oxygen in the cardiotoxicity of adriamycin. 2. The combination of Herceptin with taxol or adriamycin is relatively toxic in cardiomyocytes. Based on these observations, we propose to further explore the basis for adriamycin cardiotoxicity as well as examining the capacity of sildenafil to protect the heart cells from the combination of Herceptin with Adriamycin and with taxol. We will evaluate the influence of sildenafil on autophagy and senescence in both breast tumor cells and cardiomyocytes. We will pursue the unexpected finding that sildenafil appears to increase sensitivity to radiation in the breast tumor cells by attempting to understand the mechanistic basis for this observation. We will also assess the impact of sildenafil on radiation sensitivity in the cardiomyocyte. These proposed studies are of significance because the heart is known to incur damage during irradiation of the breast during therapy for breast cancer.					
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## INTRODUCTION

Our proposed work was based on the observations that phosphodiesterase 5 inhibitors, specifically the erectile dysfunction drugs such as sildenafil, could protect cardiomyocytes and the heart from the toxicity of the anthracycline antibiotic, adriamycin (1). Furthermore, we had generated preliminary data that sildenafil did not appear to protect breast tumor cells from adriamycin and, in fact, may have promoted sensitivity to this drug. Our research goals were to extend these observations and to delineate the mechanistic basis for these differential effects in the tumor and in the heart.

## BODY

In our current work, we have continued to explore the interaction(s) between sildenafil and adriamycin in both breast tumor cells and cardiomyocytes.

### Studies in Breast Tumor Cells

In Figure 1, we repeated studies combining sildenafil with adriamycin (doxorubicin) in MCF-7 breast tumor cells as well as MCF-7 cells engineered to express the executioner caspase, caspase 3, associated with promotion of apoptosis (2,3) and in p53 mutant MDA-MB231 breast tumor cells. However, in this work, we utilized a different exposure protocol, chronic exposure to low drug concentrations rather than acute exposure to a high drug concentration. The chronic low exposure protocol simulates the long term drug levels observed in the plasma subsequent to a pulse administration of drug. As with the acute exposure studies, sildenafil failed to protect against the antiproliferative effects of adriamycin. In fact, in all cases, we observe somewhat increased sensitivity for the combination treatment. These observations further support the potential utility of using sildenafil (or other phosphodiesterase 5 inhibitors) as cardioprotective agents when breast cancer is treated with doxorubicin. Further studies will evaluate the basis for the enhanced response to the combination treatment in terms of such factors as increased DNA damage and decreased DNA repair as well as the generation of specific reactive species.

We also assessed the effects of sildenafil on the impact of adriamycin on cell cycle distribution. Adriamycin produced an increase in the G2/M cell population, consistent with previous reports from our laboratory (4). Sildenafil failed to influence the impact of adriamycin on cell cycle distribution (data not shown), which is what would be expected given its lack of interference with breast tumor cell sensitivity to adriamycin.

Since the induction of DNA damage is thought to be a primary mode of action of adriamycin (5), we evaluated the effects of adriamycin alone as well as with sildenafil, on gamma H2AX formation, a well established indicator of DNA damage (6). Figure 2 indicates that sildenafil failed to modify the extent of damage, which is again consistent with the lack of attenuation of drug sensitivity.

Adriamycin also has the capacity to promote senescence (7) in breast tumor cells and our current studies indicate that another mode of cell death, known as autophagy (8) (Figure 3) is also evident in breast tumor cells treated with adriamycin. Studies are currently in progress to determine the impact of sildenafil on both senescence and autophagic responses to adriamycin in the breast tumor cell.

Finally, we evaluated the impact of sildenafil on irradiation of the breast tumor cell, given that breast cancer patients frequently receive radiation therapy. Figure 4 indicates that the sildenafil treatment also failed to protect the breast tumor cells against the impact of radiation and, as was the case with adriamycin, increased its effect against the breast tumor cell. Studies are in progress to assess the effects of sildenafil on the promotion and repair of DNA damage in breast tumor cells by irradiation (using the gamma H2AX assay as well as alkaline unwinding). It will also be of interest to determine whether sildenafil might protect cardiomyocytes against radiation, given that the heart frequently receives collateral radiation damage when the breast is irradiated.

### Studies in Cardiomyocytes

In terms of exploring the mechanisms whereby sildenafil might protect only cardiomyocytes while allowing adriamycin to target the breast tumor cell, it is important to recognize that the foundation for the differential effects is likely to be doxorubicin acting as a topoisomerase II poison in tumor cells (5) while its toxicity to the heart is through the generation of free radicals (9, 10). However, our studies raise some questions relating to this paradigm.

The experimental model system for these studies has been the H9c2 cardiomyocyte cell line. This is an embryonic cell line that replicates in culture and which has been used by a number of investigators as a model of cardiac function (11).

Figure 5 indicates that sildenafil failed to protect the cardiomyocytes against the impact of adriamycin in a clonogenic survival assay. Furthermore, sildenafil also failed to protect the cardiomyocytes from apoptosis. Figure 6 presents data from the TUNEL assay, demonstrating the promotion of apoptosis by adriamycin both in the absence and presence of sildenafil. These studies do raise some questions as to the validity of the findings relating to sildenafil cardioprotection in rodent models, although clearly an intact animal is likely to be a more physiologically relevant model than cardiomyocytes in cell culture.

Figure 7 indicates that adriamycin generates reactive oxygen in the cardiomyocyte, based on a 2',7'-dichlorodihydrofluorescein (DCF) staining assay, as shown by other investigators (10). However, while N-acetyl cysteine, a scavenger of free radicals, clearly reduced the extent of reactive oxygen generation, it failed to protect against the toxicity of adriamycin (Figure 8). In contrast, NAC was quite effective in protecting the cardiomyocytes against the toxicity of H<sub>2</sub>O<sub>2</sub> (Figure 9). These findings raise questions relating to the basis for adriamycin cardiotoxicity, which we propose to explore as this project progresses.

In view of the fact that treatment of breast cancer frequently involves drug combinations including taxol and Herceptin, we evaluated the combinations of Herceptin/adriamycin and Herceptin/taxol in the cardiomyocyte. Figures 10 and 11 indicate that these combinations do suppress growth of the cardiomyocytes. We will continue to assess the effects of these combinations in additional toxicity assays. Furthermore, studies are in progress to evaluate the impact of sildenafil as well as free radical scavengers on these drug combinations.

Additional experiments we propose include assessment of the impact of other free radical scavengers as well as the cardioprotectant ICRF-187 in both breast tumor cells and

cardiomyocytes. Studies are in progress to assess whether sildenafil protects macrophages (12), from drug toxicity. In addition, studies are ongoing in bone marrow cells to ascertain the impact of sildenafil on bone marrow toxicity of adriamycin.

#### KEY RESEARCH ACCOMPLISHMENTS

1. Sildenafil fails to protect various breast tumor cell lines against the toxicity of adriamycin.
2. Sildenafil moderately enhances the response to adriamycin in breast tumor cells.
3. Sildenafil does not alter the extent of DNA damage induced by adriamycin in breast tumor cells.
4. Adriamycin promotes autophagy in breast tumor cells.
5. Sildenafil appears to increase sensitivity to irradiation in breast tumor cells.
6. Questions are raised relating to the role of reactive oxygen in the cardiotoxicity of adriamycin.
7. The combination of Herceptin with taxol or adriamycin is relatively toxic in cardiomyocytes.

#### REPORTABLE OUTCOMES

Gewirtz DA. The role of senescence in cancer chemotherapy. *Current Opinions in Investigational Drugs* (in press).

Gewirtz DA, Holt SE and Elmore, LW. The accelerated senescence response to chemotherapy and radiation. *Biochemical Pharmacology* (in press).

Di X, Bright AT, Bellott R, Gaskins E, Robert J, Holt S, Gewirtz D, Elmore L. A Chemotherapy-Associated Senescence Bystander Effect in Breast Cancer Cells. *Cancer Biol Ther*. 2008 Mar 6;7(6) [Epub ahead of print]

Support of Xu Di towards PhD degree.

#### CONCLUSIONS

1. Sildenafil fails to protect breast tumor cells from either adriamycin or radiation. In fact, sildenafil appears to sensitize breast tumor cells to these as well as other treatment modalities. Consequently, this work continues to support the potential utility of sildenafil as a cardioprotectant.

2. Cardioprotection is unlikely to be related to reactive oxygen generation. The role of reactive oxygen in the cardiotoxicity of adriamycin requires further examination.

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SUPPORTING DATA

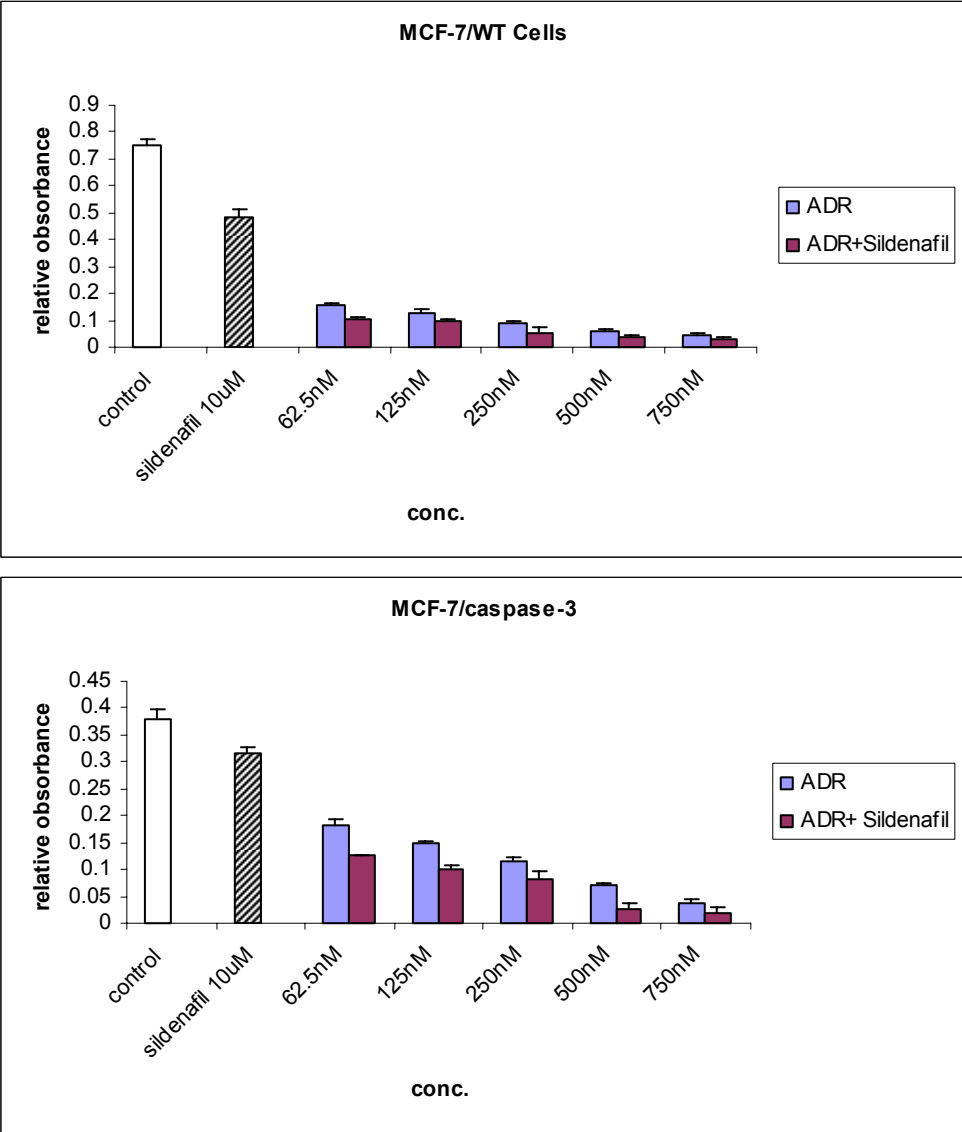
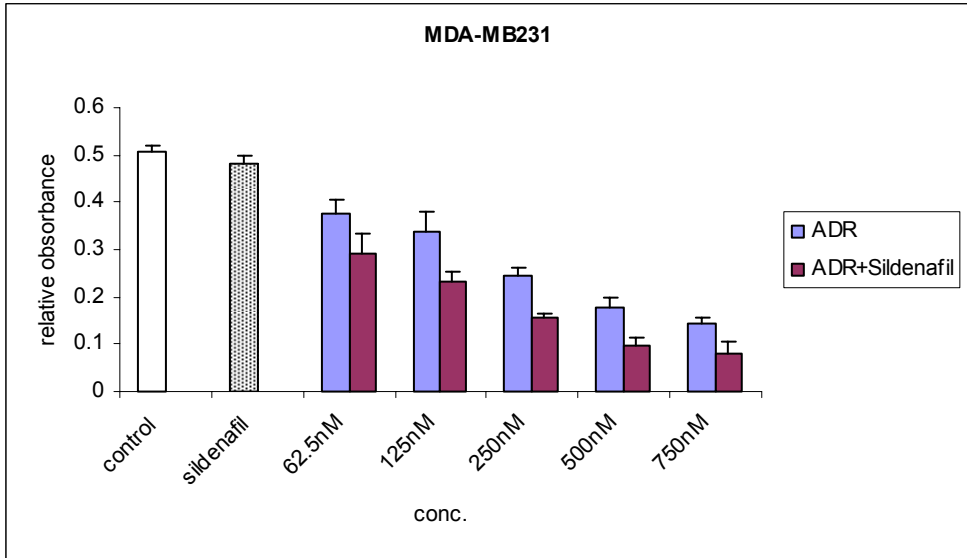
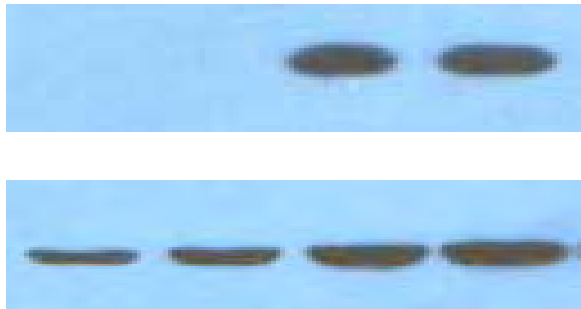


Figure 1: Lack of protection (and modest sensitization) of MCF-7, MCF-7/casp 3 and MDA-MB231 breast tumor cells from adriamycin by sildenafil. Cells were exposed to sildenafil (10µM) for 1 hour prior to incubation with adriamycin (various concentrations) for 72 hrs. Relative absorbance was measured by the MTT assay and indicates the number of viable cells.





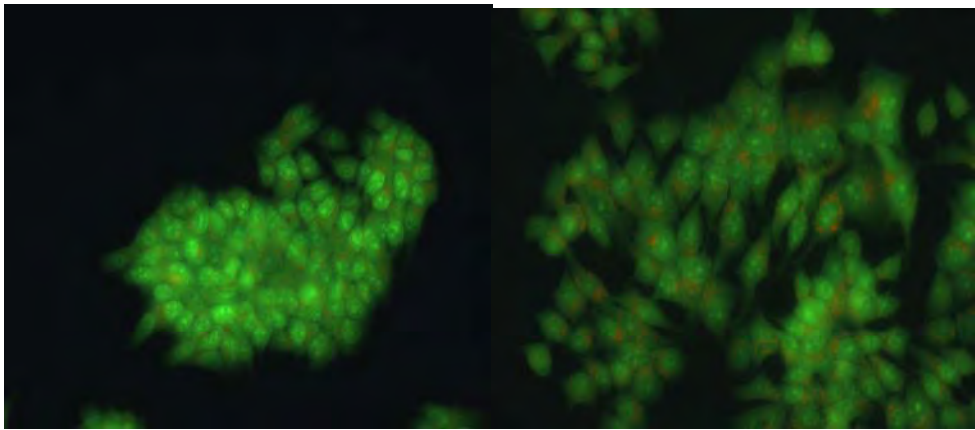
Sildenafil	-	+	-	+
AdR	-	-	+	+



$\gamma$ -H2AX

$\beta$ -Actin

Figure 2: Sildenafil does not influence the extent of DNA damage induced by adriamycin. Cells were exposed to sildenafil (10 $\mu$ M) for 1 hour prior to incubation with adriamycin (0.75 $\mu$ M) for 4 hrs.



A. Control

B. Adriamycin

Figure 3. Adriamycin promotes autophagy in MCF-7 breast tumor cells. Cells were stained with acridine orange dye. The image on the left is of control cells while the image on the right is of cells treated with 0.75uM adriamycin for 4 hours.

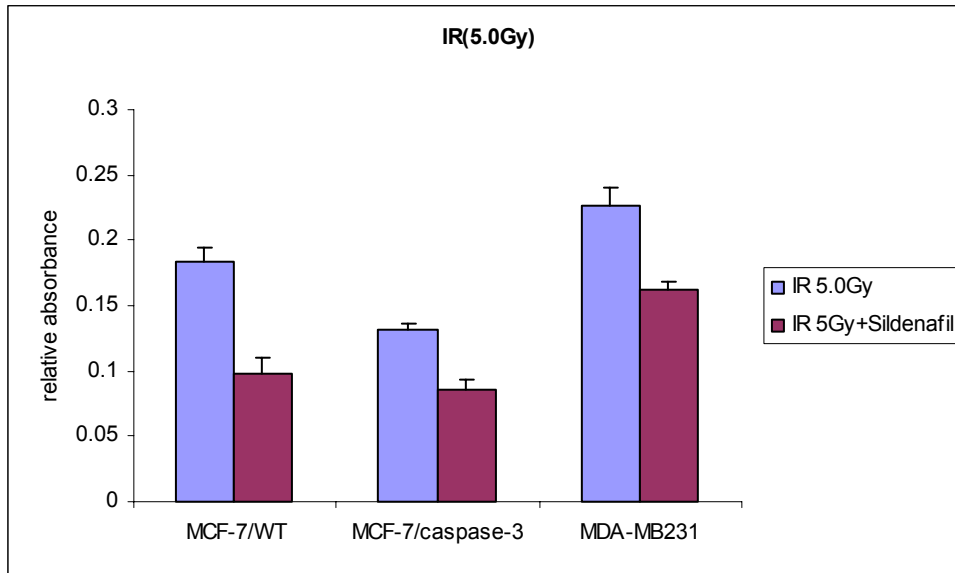


Figure 4: Sensitization of breast tumor cells to irradiation. Cells were exposed to sildenafil (10 $\mu$ M) for 1 hour prior to 5.0 Gy of irradiation. Relative absorbance was measured by the MTT assay and indicates the number of viable cells 3 days after irradiation.

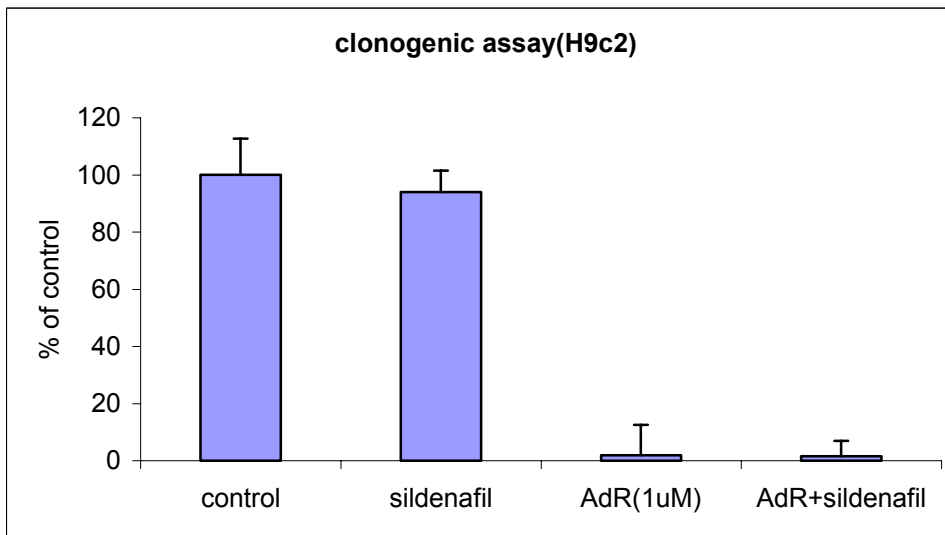


Figure 5: Sildenafil does not protect cardiac myocytes from Adriamycin in a clonogenic survival assay. Cells were exposed to sildenafil for 1 hour prior to adriamycin (0.75 $\mu$ M) for 2 hrs. Clonogenic survival was assessed after 12 days

Adriamycin (TUNEL)    DAPI stain

Adriamycin + sildenafil (TUNEL)    DAPI

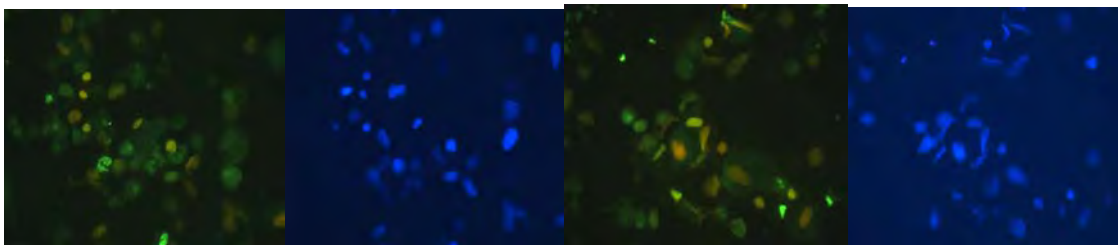


Figure 6: Lack of protection of cardiomyocytes from apoptosis induced by adriamycin. Cells were exposed to sildenafil (10 $\mu$ M) for 1 hour prior to incubation with adriamycin for 48 hrs. Apoptosis was monitored using the TUNEL assay. DAPI staining is included to indicate the presence of cells in the TUNEL stained population.

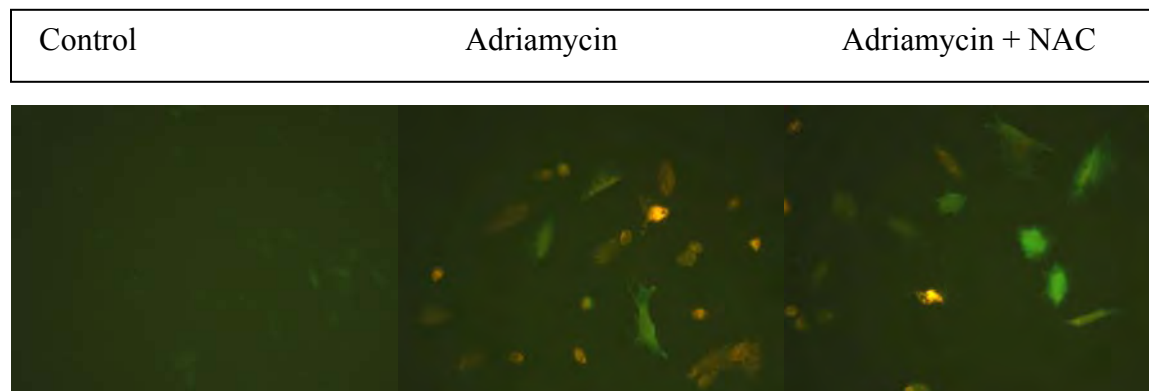


Figure 7. Dye assay for detection of reactive oxygen generation. H9C2 cardiomyocytes H9c2 were incubated with 100  $\mu$ M NAC for 2hr and treated with 2  $\mu$ M Adriamycin for 48hr in the presence of NAC. The cells were stained with 5  $\mu$ M of DCF-DA for 30min followed by fluorescent microscopy.

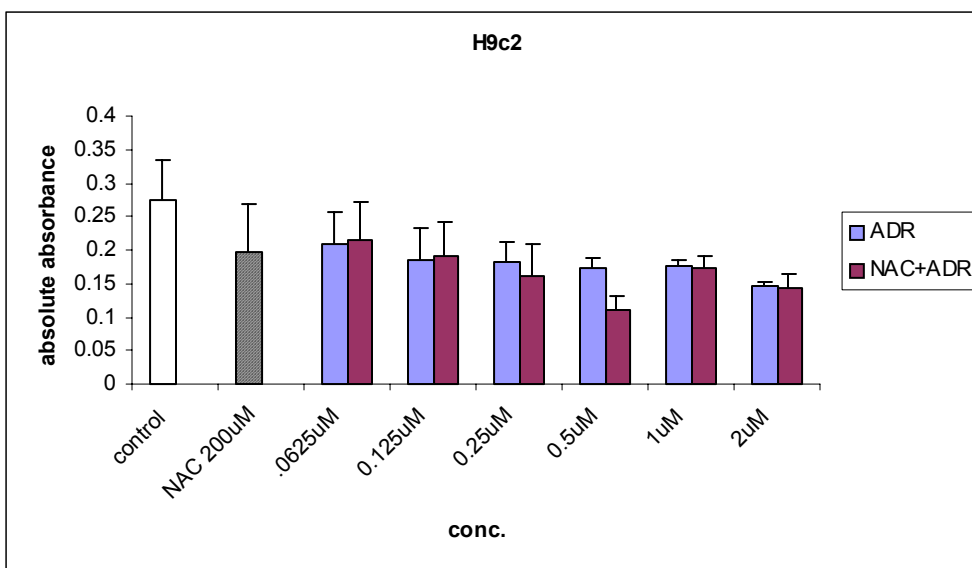


Figure 8: Lack of protection of cardiomyocytes from cytotoxicity of adriamycin by NAC. Cells were exposed to NAC (200  $\mu$ M) for 3 hrs prior to continuous incubation with different concentrations of adriamycin for 72 hrs. Viable cell number was evaluated by the MTT dye assay

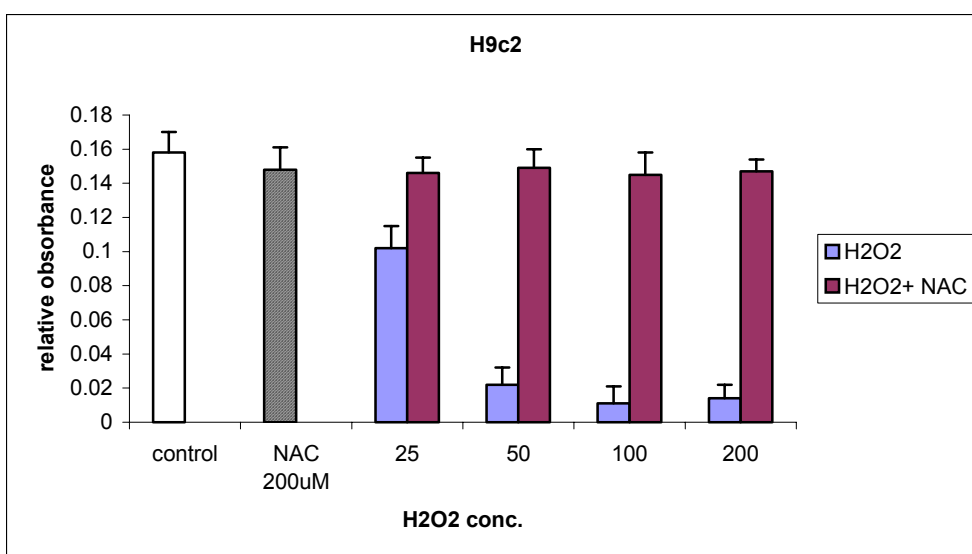


Figure 9: Protection of cardiomyocytes from cytotoxicity of hydrogen peroxide by NAC. Cells were exposed to NAC (200)  $\mu$ M for 3hrs prior to incubation with different concentrations of adriamycin for 72hrs. Viable cell number was evaluated by the MTT dye assay.

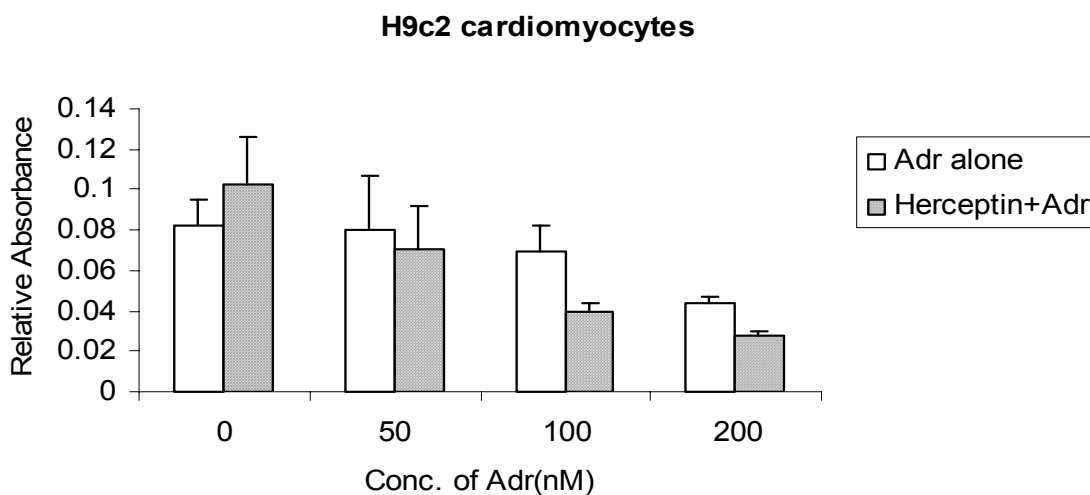


Figure 10: Antiproliferative effects of the combination of herceptin with adriamycin. Cells were exposed to herceptin at a concentration of 3.4ug/ml for 3hours followed by different concentrations of adriamycin at for 72 hours. Viable cell number was determined using the MTT dye assay.

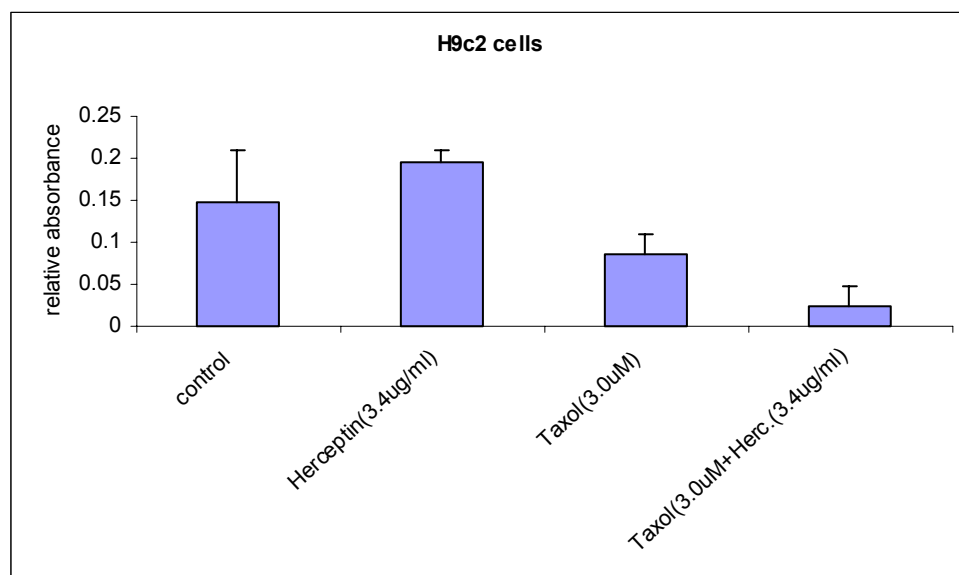


Figure 11: Antiproliferative effects of the combination of herceptin with Taxol. Cells were exposed to herceptin at a concentration of 3.4ug/ml for 3hrs followed by taxol at 3.0  $\mu$ M for 72 hours. Viable cell number was determined using the MTT dye assay.